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Importância da lisogenia no ambiente marinho

Importance of lisogeny in the marine environment

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Professora Doutora Adelaide Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Virus, lisogenia, bacterias, SML, UW, mitomicina C, diversidade bacteriana, microscopia de epifluorescência.

Resumo

Um dos papéis mais importantes dos vírus em sistemas aquáticos é a sua capacidade de agir como vectores para a transferência de genes, sendo a lisogenia o mecanismo-chave neste processo. A lisogenia pode ajudar os vírus a sobreviver a períodos de reduzida abundância de hospedeiro e / ou escassez de nutrientes, contribuindo também para o aumento de “fitness” do hospedeiro. Devido à sua localização, na interface entre a hidrosfera e a atmosfera, a microcamada superficial está exposto a elevada intensidades de radiação solar, elevadas concentrações de poluentes e metais pesados e flutuações de temperatura e salinidade. Representa, portanto, um ambiente de stress para os microrganismos, pelo que estes poderão ter desenvolvido estratégias adaptativas à sobrevivência neste microhabitat, nomeadamente a lisogenia. Por outro lado, sendo a radiação UV um importante indutor da lisogenia, a sua elevada intensidade na microcamada poderá resultar numa maior frequência de células lisogénicas neste compartimento.

O objectivo deste trabalho foi o estudo da importância da lisogenia na microcamada superficial e água subsuperficial na zona marinha e salobra da Ria de Aveiro (Portugal), tendo a fracção de células lisogénicas sido determinada após indução dos profagos com mitomicina C. Neste estudo também foi quantificado o número de bactérias que são contados como vírus quando a abundância viral é determinada por microscopia de epifluorescência.

A percentagem de células lisogénicas na microcamada superficial da zona marinha variou entre 1,2% e 3,1% e na água subsuperficial entre 1,0% e 5,3%. Na zona salobra, a proporção de células lisogenicas na SML variou entre 0,9% e 6,0% e na coluna de água entre 1,0% e 4,7%. A fracção de bactérias lisogénicas foi semelhante na microcamada superficial e na água subjacente. Não foi observado um perfil de variação sazonal nítido para a lisogenia, mas a fracção de bactérias lisogénicas foi maior, em ambos os compartimentos, quando as condições ambientais foram mais adversas. Os perfis de DGGE mostraram que alguns grupos de bactérias desapareceram após a indução da lisogenia, mas outros grupos de bactérias, não observados no controle, apareceram após a adição da mitomicina. Na zona marinha do sistema estuarino da Ria de Aveiro 27% das partículas contadas como vírus são bactérias, mas na zona salobra apenas 14% dessas partículas são bactérias.

Embora a ocorrência de lisogenia no sistema estuarino da Ria de Aveiro não seja muito alta, a variação sazonal da fracção de bactérias lisogénicas sugere que a lisogenia pode ser influenciada por variações de temperatura, salinidade ou intensidade de radiação UV. Quando a microscopia de epifluorescência, é usado para contar partículas virais, a abundância viral pode ser sobrestimada, nomeadamente na área marinha.

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Keywords

Virus, lysogeny, bacteria, SML, UW, Mitomycin C, bacterial diversity bacteriana, epifluorecence microscopy.

Abstract

Acting as gene transfer vectors constitutes one of the main roles played by viruses in aquatic systems, being lisogeny a key mechanism in this process. Lisogeny can help viruses withstand low host abundance periods and/or nutrient limitation, potentially contributing to increased fitness of the host as well. Due to its location, at the air-water interface, the surface microlayer (SML) is exposed to high intensities of solar radiation, enhanced concentrations of pollutants and heavy metals and strong temperature and salinity fluctuations. Therefore, it represents a stressful environment for microorganisms, which might have developed adaptative strategies for survival at this interface, including the presence of prophages. On the other hand, as UV radiation is an important lisogeny inducer, intense UV levels at this layer might result in increased lisogenic cell frequency in the SML. The aim of this work was to study the role of lisogeny at the SML and underlying waters (UW) of the marine and brackish water sections of Ria de Aveiro (Portugal), using the mitomycin C method to induce prophage. In this study was also quantified the number of bacteria that are counted as viruses when viral abundance is determined by epifluorescence microscopy. The proportion of lisogenic bacteria in the marine zone ranged from 1.2% to 3.1% at the SML and from 1.0% e 5.3% in the UW. At the brackish water site, the fraction of lisogenic bacteria ranged from 0.9% to 6.0% at the SML and 1.0% to 4.7% at the UW. The fraction of lisogenic bacteria was similar in SML and in UW. It was not observed a clear pattern of seasonal variation of lisogenic bacteria, but the high values of lisogeny were observed, for both compartments, when the environmental conditions were more adverse. The DGGE profiles show that some groups of bacteria disappeared after the induction of lisogeny but other groups, not detected in the controls, appears after the incubation with mitomycin.

In the marine zone of the estuarine system Ria de Aveiro 27% of particles counted as viruses are bacteria but in the brackish water zone only 14% of those particles are counted as viruses. Although the occurrence of lysogeny in the estuarine system Ria de Aveiro is not high, seasonal variation in the fraction of lisogenic cells suggests that lisogeny can be influenced by changes in temperature, salinity and in the intensity of UV radiation. When epifluorescence microscopy is used to count viral particles, viral abundance can be overestimated, namely in the marine area.

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List of Acronyms and Abbreviations

ANOSIM	Analysis of Similarities
BBP	Bacterial Biomass Productivity
BCC	Bacterial community composition
CFU	Colony forming units
DAPI	4', 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescent in situ hybridization
HNF	Heterotrophic nanoflagellates
L	Liter
m	Meter
MDS	Multidimensional scaling
mL	Milliliter
NaCl	Sodium chloride
nM	Nanomolar
NVP	Number viral particles
PAH	Polyaromatic hydrocarbon
PBS	Phosphate buffer system
PCR	Polymerase Chain Reaction
R	Correlation coefficient
SDS	Sodium Dodecyl Sulphate
SML	Surface microlayer
TBN	Total bacterial number
TEM	Transmission of electron microscopy
UV	Ultraviolet
UVR	Ultraviolet radiation
UW	Underlying water
µL	Microliter
µm	micrometer
µM	Micromolar

1.Introduction

1.1 Importance of bacteria in the marine environment

For many years, bacteria were not considered an essential component of the biogeochemical processes of the aquatic system. However, this view has changed and now it is well known that microorganisms play an important role in biogeochemical cycles. (Azam, Fench et al. 1983)

Bacteria are the most important biological components involved in the turnover (transformation and remineralisation) of organic matter in aquatic systems (Pomeroy *et al*., 1991) (Pommeroy 1991). Heterotrophic bacteria often represent 10-30% of the living carbon biomass (Holligan *et al*., 1984 (Holligan 1984; Cho 1990) and may utilize as much as 40% of the carbon fixed by the primary producers ((Cho 1990); (DUCKLOW 1992)By converting dissolved organic carbon into a particulate form, potentially useable by higher trophic levels, heterotrophic bacteria represent a key link in the cycles of energy and carbon in the aquatic systems, as depicted in the concept of the microbial loop proposed by Azam et al (1983). Studies in several aquatic environments have indicated that the microbial loop can process about as much energy as the classical grazing food chain (Riemann and Søndergaard 1986)However, the current description of the microbial loop is not a simple task as new players, namely viruses, have been added. The importance of viruses in the microbial loop is still uncertain, but it is known that they influence the cycle of organic matter when infect and destroy bacteria, algae and cyanobacteria. When cell lysis occurs, particulate organic matter is lost from the food chain but becomes available to heterotrophic bacteria.

1.2. Bacterioplankton abundance, diversity and production in the marine environment

During the last forty years it has become clear that bacterioplankton makes a significant contribution to planktonic abundance ((Holligan 1984): (del Giorgio and Cole 1998) and productivity (Cole 1988; Goosen 1997) in aquatic systems. Bacteria are the second most abundant biological entity (after viruses) and the first in terms of biomass

(Smith, Sorial et al. 1996),and their production has been found to average 20% of primary production ((Williams 1981); (Cole 1988)), and about twice the production of macrozooplankton (Cole *et al.*, 1988). Furthermore, microbial communities are influenced by several factors that operate with different strength in oceanic and estuarine environments. These factors act also differently within an aquatic system, creating vertical, longitudinal, seasonal and tidal patterns of variation.

The abundance and productivity of the bacterial community in aquatic systems vary according to the temperature range ((Heinanen and Kuparinen 1991; Siervi, Mariazzi et al. 1995)), depth of the water column (Pace and Cole, 1994; Talbot *et al.*, 1997 (Pace and Cole 1994; Talbot 1997) and, in estuaries, with the proximity to the sea (Almeida 1994; Goosen 1997; Konstantinos Ar, Konstantinos et al. 1998; Almeida, Cunha et al. 2001) and with tidal water circulation (Almeida et al, 2001;(Shiah 1995; Hoppe 1996)

Bacterial abundance in aquatic environments is in the range of 10^8 to10^9 cells mL^{-1} del Giorgio et al., 1996; Hoppe et al., 1996). In estuarine environments, bacterial abundance is 1 to 3 times higher in comparison with open waters (Hall and Vincent, 1990; Børsheim, 2000). Bacterial abundance in open waters shows a clear vertical profile of variation, with the density of cells decreasing below the photic zone (Bianchi and Juliano., 1996). In coastal waters the highest abundance occurs at surface waters (Heinamen, 1991; Almeida et al, 2001, Santos et al, 2009), but in shallow estuarine systems bacterial abundance can be homogenous along the water column (Ducklow and Shiah, 1993) or even higher in the bottom (Amon and Benner, 1998). In the cold seasons, total bacterial abundance decreases in open waters and in coastal waters (Hoppe, 1978). In estuarine systems, longitudinal profiles in bacterial abundance also occur, with an increase from the outer to the inner (konstantinos et al, 1998; Almeida et al, 2001; Almeida et al, 2005) or mid (Cunha et al, 2000: Almeida et al, 2001; Almeida et al, 2005) estuarine sections. In these systems, bacterial abundance increases when near low tide when compared to high tide (Almeida et al, 2001).

Only a fraction of the total bacteria is active (Almeida et al, 2001) and, consequently, the total number of bacteria may not be a useful ecological parameter, because only the active bacteria are responsible for the growth, nutrient uptake and

transformation of organic carbon substrates. This fraction of active cells can be selectively grazed (del Giorgio et al, 1996) or infected by viruses (Proctor and Fuhrman, 1990). Total bacterial number gives important information of standing stock without, however, reflecting the real metabolic activity. The number of active cells varies more than the total number among and within systems (del Giorgio and Scarbough, 1995), but the trend of the variation is similar.

In aquatic environments, bacterial communities are represented by a limited number of individual bacteria (Murray et al., 1998; Pinhassi et al., 2000). Salinity, nutrient concentration, organic matter composition and the structure of the bacteriovore community are thought to influence the composition of natural bacterioplankton communities (Crump et al, 2003). Besides salinity, geographic variability in estuarine bacterial community composition is mainly governed by dynamic events like algal blooms, temperature change and upwelling (Fukami *et al.*, 1985; Hagstrom *et al*., 2000). Seasonal variations in bacterial community composition have been attributed to changes in inorganic nutrient concentrations (Pinhassi and Hagstrom 2000) and in the nature of the dissolved organic matter (DOM) pool (Cottrell and Kirchman 2000a). Typically, estuaries are characterized by a shift in the dominant bacterial groups along the salinity gradient from α- and β-Proteobacteria, Gram-positive bacteria and Verrucomicrobia to α- and γ-Proteobacteria (Crump et al. 1999; Henriques et al, 2006). Environmental factors, such as precipitation and temperature, can also affect the estuarine bacterial community composition (Kan et al, 2006)

In aquatic systems, bacterial productivity by heterotrophic bacterioplankton is in the range of 0.0003-26.2 µg C l^{-1} h^{-1} (Hoppe *et al.*, 1998; Kisand and Noges, 1998, Shiah *et al.*, 1999; Almeida et al, 2001; Almeida et al, 2005). In estuarine systems the values are often high when compared to the adjacent coastal areas and open sea (Ducklow and Shiah, 1993; Di Siervi *et al.*, 1995; Almeida et al, 2005). The highest values have been registered during summer months and the lowest during the winter in both estuarine and oceanic waters (Di Siervi *et al.*, 1995; Almeida et al, 2002). Vertical profiles of bacterial production in oceanic waters are usually characterised by decreasing values from surface to the deeper water layers (1996; Talbot *et al.*, 1997; Almeida et al, 2001). In estuarine

systems bacterial production is, in general, higher at surface waters (Heinanen, 1991;) but in shallow estuaries bacterial productivity can be similar throughout the water column (Ducklow and Shiah, 1993) or even higher near the bottom sediment (Amon and Benner, 1998). Frequently bacterial productivity increases up to 10 times from the lower to the mid-upper sections of the estuary (Goosen *et al.*, 1997;, Cunha *et al.*, 2000; Almeida et al, 2001). In these systems, the highest values of bacterial productivity have been observed near low tide (Shiah and Ducklow, 1995; Hoppe *et al.*, 1996; Cunha *et al.*, 2000; Almeida et al, 2001).

1.3. Factors affecting bacterioplankton in the marine environment

Longitudinal profiles of bacterioplankton abundance, production and diversity in the marine environment are clear in coastal waters and result from physical, chemical, nutritional and biological pressures that are in different balance in marine and brackish water zones (Almeida et al, 2001, Henriques et al, 2004). Higher values of nutrients and low values of salinity in the mid and upper sections of estuarine systems stimulate bacterioplankton growth. However, in these estuarine sections, biological control, namely viral lysis, affects bacteria negatively (Almeida et al, 2001).

The seasonal variation of bacterioplankton depends on the region. In temperate systems, variation of bacterioplankton peaks during the warm season and the lowest values are observed during the cold season (Almeida et al, 2001,). This pattern of variation is clear in coastal waters, but oceanic environments show seasonally stable regimes. Temperature and salinity are the parameters that control this profile of variation (Shultzand Ducklow, 2000).

Bacterioplankton tidal variation is due to water circulation, which affects salinity, temperature and nutrient concentration, namely in coastal waters. Water circulation and wind can also change estuarine conditions in a smaller temporal scale, in comparison with the open ocean (Shultz and Ducklow, 2000). Clear patterns of tidal variation are observed in estuarine systems, showing increasing density, activity and diversity near low tide relatively to high tide (Almeida et al, 2001).

These factors controlling bacterioplankton can, therefore, be classified as nutritional (availability of organic and inorganic substrates), physical (e.g temperature, water circulation), chemical (e.g. salinity) and biological (predation and viral lysis).

1.3.1. Availability of organic and inorganic nutrients

It is well known that nutrients represent a major factor regulating bacterioplankton in aquatic systems (Ducklow and Carlson, 1992; Proctor, 1992; Shiah et al., 1999).

In aquatic systems, nutrients supporting bacterial heterotrophic growth can be classified according to their origin as autochthonous or allochthonous. Autochthonous nutrients are produced in the ecosystem by primary producers - phytoplankton. Phytoplankton can directly supply bacteria with organic matter, through exudation from healthy cells and lysis of senescent and dead cells (Vadstein et al., 1993; Panzenbock et al., 2000). Indirect supply occurs via viral lysis or grazing by herbivorous zooplankton (Peduzzi and Herndl, 1992; Bratbak et al., 1998; Noble and Fuhrman, 1999). However, in coastal systems, primary production may not be sufficient to support bacterial growth (Almeida et al, 2005). In these systems, bacterial growth is largely dependent on non-phytoplanktonic carbon sources, including allochthonous sources, such as river transported materials, terrestrial runoff, anthropogenic discharges, benthic fluxes and sediment resuspension (Lee et al., 2001; Almeida et al, 2001). Findlay et al. (1992) estimated that the amount of allochthonous carbon inputs needed to support bacterial productivity in the Hudson Estuary was three to six times greater than the net carbon fixed by phytoplankton. An identical trend was also observed in Lawrence Lake (Coveney and Wetzel, 1995). However, the original allochthonous compounds undergo partial degradation and transformation before entering the aquatic system and might be less labile than the autochthonous substrates originated from phytoplankton production (Hobbie, 1988). So, allochthonous organic matter can provide stability to bacterioplankton, in the sense that the large pool of recalcitrant organic matter supports

continuous slow growth, independent of the intermittent growth associated with the less constant presence of autochthonous labile organic matter (Wetzel, 1984; Hobbie, 1988).

In recent years, the theory that bacterial growth rates are dictated solely by available organic carbon has changed and the role of inorganic nutrients, such as nitrogen and phosphorus in the regulation of bacterial production in aquatic ecosystem has been recognized (Thingstad et al., 1993; Rivkin and Anderson1997; Torréton et al., 2000). In fact, several studies have shown that bacterial growth increases with increasing availability in inorganic nutrients (Kroer, 1993; Pace and Cole, 1996; Torréton *et al.*, 2000). Moreover, heterotrophic bacteria have been shown to compete successfully with phytoplankton for inorganic nutrients (Currie and Kalff, 1984; Thingstad *et al.*, 1993). Blackburn *et al.* (1998) showed that bacteria have nutrient uptake potentials around 100 times faster than that of phytoplankton. At low concentrations, the competitive advantage of bacterioplankton over phytoplankton for inorganic nutrients is a consensual idea (Dufour and Berland, 1999; Torréton *et al.*, 2000).

1.3.2 Water properties

It has been shown that bacterial growth is affected by temperature (Shiah et al., 1999), particularly in eutrophic and mesotrophic systems, where substrate availability plays a smaller role (Shiah and Ducklow, 1995). A reciprocal interaction between temperature and substrate has also been observed (Wiebe and Pomeroy 1992).

Salinity is a selecting agent that influences the bacteria that may proliferate in an ecosystem (Rheinmheirmer, 1985). Bacteria that need salt for their growth are unable to thrive in estuarine environments with strong freshwater inputs (Campbell, 1983). In contrast, it is unlikely that inflowing freshwater bacterial populations may survive within an estuary (Valdés and Albright, 1981; Painchaud et al., 1987). In estuarine systems bacterioplankton exhibit a higher abundance and activity at low salinities (Murrel et al., 1999), with decreasing values towards higher salinity values (Murrell et al., 1999).

Although there are only a few reports of light effects on bacterioplankton, photoinhibion of heterotrophic bacteria has been reported (Pakulski et al., 1998; Pausz

and Herndl, 1999; Mousseau et al., 2000). For example, the bacterial production in the Adriatic Sea surface was inhibited by as much as 40% by UV-radiation (Mousseau et al., 2000). Bacterial photoadaptation does not seem to occur, since bacteria below the photic zone are as sensitive to light as bacteria in the euphotic zone (Lindelland Edling, 1996). Although photoinhibition results in decreased bacterial activity, photolysis of recalcitrant organic matter can actually stimulate bacterial growth (Bushaw-Newton and Moran, 1999). In surface waters, photochemical transformation of recalcitrant organic matter into labile compounds can occur, resulting in enhanced local and transient growth of active bacteria (Bano et al., 1997; Bushaw-Newton and Moran, 1999). Organic matter exposed to natural solar irradiation enhanced 11-13 times the activity of bacterial community of the estuarine system Ria de Aveiro (ongoing PhD thesis). However, organic matter phototransformation can also lead to the formation of photoinhibitory compounds, such as reactive oxygen species and free radicals (ROS) that may inhibit the activity of bacterioplankton.

Light effects on phytoplankton and on virioplankton can also influence bacterioplankton growth. UV-radiation can reduce phytoplankton photosynthesis (Helbling *et al.*, 1996a) and growth (Jokiel and York, 1984), enhance phytoplankton exudation (Zlotnik and Dubinsky, 1989; Feuillade *et al.*, 1990) and even modify the structure of the phytoplankton community (Helbling *et al.*, 1996b). Solar radiation is a major cause of decline in viral infectivity in surface waters (Noble and Fuhrman, 1997), reducing virus-mediated mortality of bacterioplankton.

1.3.3. Water circulation

Water circulation in estuarine environments is capable of changing estuarine conditions in a much smaller temporal scale than that occurring in the open ocean (Shultz and Ducklow, 2000). The abundance and productivity of bacterial communities in the estuarine environment can be affected by water circulation, as it supplies allochthonous organic matter and concentrates locally produced organic matter within the estuary (Ducklow and Shiah, 1993).

The water circulation in estuaries is forced by riverine inflow, tides, rainfall and evaporation (Wolanski, 2007). Tidal currents interfere with vertical distribution patterns while tidal currents can destratify the water column (Haas, 1977), stimulating total bacterial production (Ducklow, 1982).

1.3.4. Mortality through predation and viral infection

One of the major factors of mortality in aquatic bacterial communities is predation (bacterial mortality of 5 to 250% per day) (Weinbauer and Hofle, 1998; Weisse and Muller, 1998). Heterotrophic nanoflagellates (HNF) and ciliates are often considered the major predators of bacteria (e.g. Sanders et al. 1989, 2imek et al. 1990, Berninger et al. 1991). Moreover, many experimental studies have shown that protista predation and zooplankton grazing changes bacterioplankton communities in terms of size structure and community composition (Chrzanowski & Simek 1990, Jürgens 1994, Hahn et al. 1999, Simek et al. 1999). Selective protista bacterivory has been suggested as one of the key factors regulating natural bacterial community composition (BCC) (Jürgens et al. 1999, Simek et al. 1999, 2001b, Langenheder & Jürgens 2001). However, the interplay of selective and unselective predation is probably quite complex and changes in the grazer community may have varying effects on BCC (Simek et al., 2003). Some studies have shown the occurrence of highest rates of grazing on active (del Giorgio et al., 1996) and motile bacteria (Gonzalez et al., 1993). It has also been shown that bacteria and heterotrophic nanoflagellates (HNF) are not strongly coupled across systems, and, consequently, HNF do not always control bacterial abundance, probably because of predatory control of HNF by larger zooplankton (e.g., daphnids) . Ducklow and Carlson (1992) have argued that the control mechanisms may also change seasonally.

In the late 80´s it was concluded that the majority of viruses are bacterial viruses (bacteriophages) and that viral lysis is a major cause of bacterial mortality (Weinbauer M. and Hofle M 1998) contributing also to the regulation of bacterial production (Weinbauer M. and Hofle M 1998). In many marine environments, the effect of viral lysis on bacterial mortality has been compared with the one of flagellate grazing and the proportion of

bacterial production removed by viral lysis can be as high as the proportion removed by grazing. Viruses can transform microbial biomass into dissolved and particulate organic matter (Figure 1) by lysis and export more carbon and other organic molecules out the water column by the accelerated sinking rates of virus-infected cells. Accelerated sinking, as the result of viral infection, might be a mechanism that enhances the export of the smallest primary producers from surface waters (Sutlle, 2005).

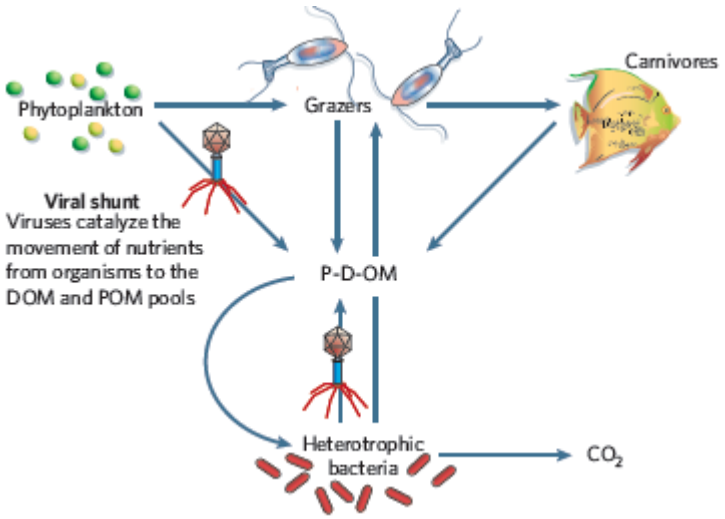


Figure 1 Viruses are catalysts for biogeochemical cycling (Suttle, 2005).

Viruses can influence bacterial diversity at the genetic level since they can mediate genetic exchange via transduction (Sayler and Miller, 1992; Paul, 1999). In this process, bacterial genomic DNA or plasmid DNA is encapsulated into phage particles during lytic replication of the phage in the donor cell and is transferred to the recipient cell by infection. This donor DNA either undergoes recombination with the host chromosome to produce a stable transductant or remains extrachromossal as a plasmid (Cochran and Paul 1998). Viruses can also influence the structure of bacterial communities due to their host specificity (Wommack et al., 2000).

1.4. Viruses and lisogeny in the marine environment

The world of prokaryotic viruses, including bacteriophages (phages) and the viruses of Archaea, is currently in a period of renaissance, due to metagenomic

sequencing advances and the isolation of diverse novel virus-host systems (Comeau et al, 2008). The resurgence of interest in prokaryotic viruses began in the mid 90s as a consequence of their extraordinary abundance in the marine environment, and of the unchallenged acceptance of the fact that viruses represent the greatest pool of genetic diversity on the planet (Angly et al, 2006; Culley et al, 2006).

Viruses are, by far, the most abundant biological entities in the aquatic systems (Fuhrman, 1999; Wommack and Colwell, 2000;) and their enormous abundance (around 10^{10}-10^{11} particles L^{-1} of water) and vast diversity still need more studies to provide the vital clues to their real function in natural ecosystems. The estimation of 10^{30-31} viruses in marine waters (Wommack and Colwell, 2000; Suttle, 2005) corresponds to 10^{23-25} viral infections per second (Pedulla et al, 2003; Suttle, 2007). Most marine viruses are bacteriophages that kill bacteria (Weinbauer, 2004), influencing the species composition of microbial communities (Wommack and Colwell, 2000). They have a restricted range of host cells and, consequently, infection by a particular virus does not act on total microbial assemblage but rather on specific sub-populations. Viral lysis in surface waters removes 20-40% of the standing stocks of prokaryotes each day (Suttle, 1994) and can match grazing by protists as a source of mortality of bacteria (Fuhrman and Noble, 1995; Almeida et al, 2001; Weinbauer, 2004). However, since these lysis products are readily utilizable by bacteria, viral lysis can actually stimulate bacterioplankton (Middelboe et al., 1996). Consequently, viral lysis plays a significant role in the cycling of nutrients and organic matter (Fuhrman, 1999).

In the marine environment most phages are dsDNA tailed, belonging mainly (96% of the total) to the Caudoviridales order (families Myoviridae, Siphoviridae and Podoviridae), but there are also small groups with ssDNA, ssRNA or dsRNA (Table 1) that can be important in the marine environment. Metagenomic approaches have shown that a large number of sequences (6% of the total) correspond to ssDNA phages belonging to Microviridae family (Angly et al, 2006). RNA phages are also present in the marine environment (Børsheim, 1993; Alcântara et al, 1995; Grabow, 2001; Cole, 2003) but in a recent metagenomic analysis of coastal waters, no RNA phages were detected (Culley et al, 2006). However, Culley *et al.* (2006) showed that the marine environment is a

reservoir of previously unknown RNA viruses, revealing that 98% of RNA viruses belong to positive-sense ssRNA viruses. In the same study, however, most of the RNA phages were classified as unknown and maybe some of them are RNA phages, since there are only a few number of viral RNA sequences in the databases, which difficult viral diversity interpretation.

Table 1 Classification and basic properties of bacteriophages (Ackermann, 2003)

Shape	Nucleic Acid	Order and families	Genera	Members	Characteristics
Tailed	DNA, ds, L	*Caudovirales*	15	4950	
		Myoviridae	6	1243	Tail contractile
		Stphoviridae	6	3011	Tail long, noncontractile
		Podoviridae	3	696	Tail short
Polyhedral	DNA, ss, C	*Microviridae*	4	40	
	ds,C,T	*Cortocoviridae*	1	3?	Complex, capsid, lipids
	ds, L	*Tectiviridae*	1	18	Internal lipoprotein vesicle
	RNA, ss, L	*Leviviridae*	2	39	
	ds,L, S	*Cystoviridae*	1	1	Envelope, lipids
Filamentous	DNA, ss, C	*Inoviridae*	2	57	Filaments or rods
	ds,L	*Lipotrixviridae*	1	6?	Envelope, lipids
	ds,L	*Rudiviridae*	1	2	Resembles TMV
Pleomorphic	Dna, ds, C, T	*Plasmaviridae*	1	6	Envelope, lipids, no capsid
	ds, C, T	*Fuselloviridae*	1	8?	Spindle-shaped, no capsid

C, circular; L, linear; S, segmented; T, superhelical; 1, single-stranded; 2, double-stranded.

Viruses can interact with their hosts in two major and distinctive ways, the lytic and lysogenic cycles of infection. More sporadically, the interaction might also proceed through pseudolysogeny. In the lytic cycle, the phages (so-called lytic or virulent) redirect the host metabolisms towards the production of new phages, which are released during the lysis of the cell. In the lysogenic cycle, the genome of the phage (temperate or lysogenic) typically remains in the host in a dormant stage (prophage) and replicates along with the host, until the lytic cycle is induced. A lysogenic decision (Figure 2), whether or not to establish a prophage state, is made by the temperate phage after infection. Lisogeny might be a viral survival strategy to endure periods of low host density during nutrient starvation (Freifelder, 1983; Wilson and Mann, 1997). Lysogenic bacteria may also gain specific advantages from their relationship with phages that improve their overall fitness. These effects may occur through the process of conversion, whereby

prophage genes are expressed in the lysogen, resulting in expanded metabolic capabilities, antibiotic resistance and toxin production. Most frequently, prophage gene expression leads to homoimmunity (Levin et al, 1983) that provides resistance to superinfection by the same or similar strains of phages. There are some classical data referring that *Escherichia coli* cells containing prophages grew quicker than nonlysogenic *E. coli* (Edlin et al, 1977). The lytic cycle is induced by physical or chemical agents, such as radiation, pollutants and changes in temperature, salinity and nutrient concentration (Cochran et al, 1998; Weinbauer and Suttle, 1999).

Pseudolysogeny (i.e., false lisogeny) is described as a phenomenon where there is a constant production of phage in the presence of high host cell abundance (Ackermann and DuBow, 1987). It has been considered an environmental condition resulting from bacterial nutrient deprivation coexisting in an unstable relationship with infective viruses (Ripp and Miller, 1998). Under such condition, host cells do not provide enough energy in order for the phage to enter in a true lysogenic or lytic condition. The phage lysis results not in total host death, but rather in a state in which a high abundance of phage coexists with exponential host growth. Once the bacterial starvation condition is relieved, the bacteriophage can either proceed with lytic infection or enter a dormant intracellular phase (Wommack and Colwell, 2000).

1.5. Life cycle steps of lytic and lysogenic cycles

Several steps in the process of phage replication are common to all viruses. The first step of the infection is the adsorption of the phage to the bacterial cell, which is reversible and represents a possibility of the phage to decide against infection (Weinbauer, 2004). The second step is the formation of an irreversible attachment of the phage to the bacteria that is mediated by a phage structure (e.g tail fibers). After these first steps, the cell wall is made penetrable (e.g., by special phage enzymes in the tail or capsid) and the viral nucleic acid is transported into the cell, whereas the capsid remains outside the cell. Following injection, the genetic material is either integrated into the host genome or stays in the cytoplasm (Weinbauer, 2004).

In the lytic cycle, before the lysis, there is an eclipse period where no infectious phage particles can be found either inside or outside the bacterial cell. The phage nucleic acid takes over the host biosynthetic machinery and phage-specific mRNA and proteins are made, in an orderly fashion. Early mRNA code for early proteins needed for phage DNA synthesis and for shutting off host DNA, RNA and protein biosynthesis. After the phage DNA is made, late proteins, comprising the structural proteins that constitute the phage and the proteins needed for the bacterial lysis are synthesized. Newly synthesized nucleic acids and proteins are then assembled and infectious phage particles accumulate within the cell; this accumulation induces the beginning of cell lysis. The number of particles released per infected bacteria may be as high as 1000 (Murray et al., 2005).

In the lisogenic cycle, after entrance of the phage DNA in the cell, a phage-coded enzyme catalyzes the recombination between a particular site on the circular phage DNA and a particular site on the host chromosome. The result is the integration of the phage DNA into the host chromosome. The second event that leads to lisogeny starts when the phage expresses the protein CI, that the repressor that binds to a particular site on the phage DNA, the operator. The repressor shuts off transcription of most phages genes except the repressor gene. During the lysis-lisogeny decision, the protein CI is expressed from a promoter termed Pre, in the lysogenic state, CI is expressed from a different promoter, termed Prm (promoter for repressor maintenance), wich maintains the lysogenic state (Waldor et al., 2005).

Although the lysogenic state is highly stable, a lysogen can switch to the lytic state. The termination of lisogeny is called induction. Induction can be cause by desiccation, exposure to UV or ionizing radiation, as well as exposure to mutagenic chemicals and heat .Mitomycin c is an antibiotic of the family of aziridine and represents, along with UV-C radiation (<300 nm), one of the most powerful inducing agents (Weinbauer, 2004). When the process of induction starts, a protease (Rec a) is produced, and cleaves the repressor protein, giving rise to the beginning of the lytic cycle (Figure 2) (Murray et al. 2005; Waldor et al., 2005). The decision for a phage to enter the lytic or lysogenic cycle is taken when the virus enters the cell. The decision is based on the concentration of the repressor and the phage

protein Cro present in the cell (Waldor et al., 2005). The Cro protein turns off the synthesis of the repressor and thus prevents the establishment of lisogeny. Environmental conditions that favor the production of Cro will lead to the lytic cycle, while those that favor the production of the repressor will lead to lisogeny.

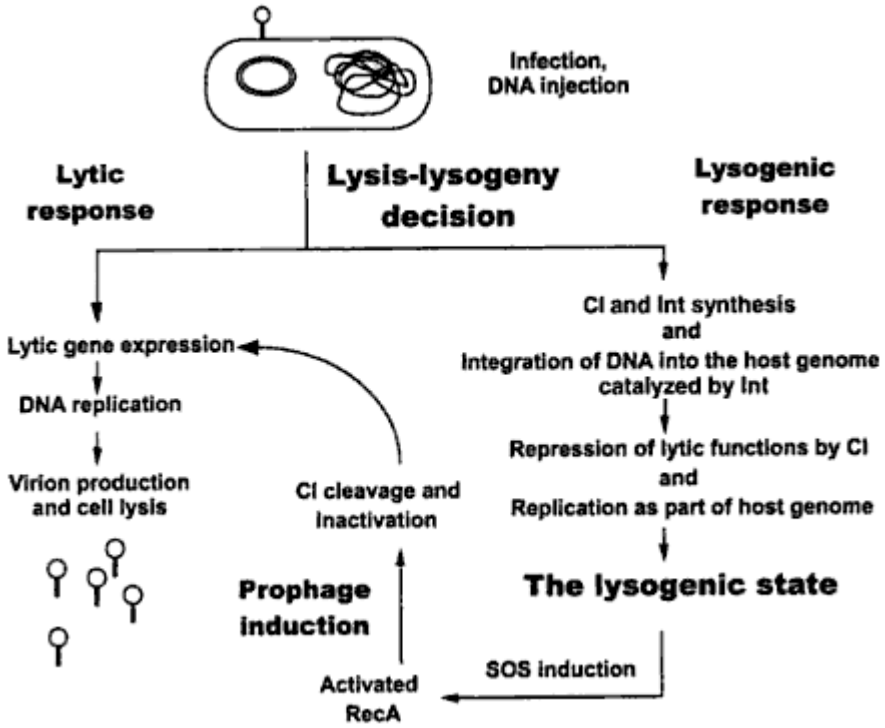


Figure 2. Life cycle of phage λ. An infected cell is depicted at the top, in which injected phage DNA has rapidly circularized. Ten to 15 min after infection, a decision made between two alternatives fates (Waldor et al., 2005).

1.6 Role of Virus in Aquatic Ecosystems

The prevalence of lytic and lysogenic infection in the marine environment is a topic of considerable debate. According to Freifelder (1983), over 90% of known bacteriophages are temperate, but other authors (Ackermann and DuBow, 1987; Cochran et al, 1998; Jiang and Paul, 1994) suggested that only around 50% of bacterial strains contained inducible prophages. This variation can be, in part, attributed to marine environment characteristics, since the prevalence of lisogeny has been shown to be highly variable across aquatic environments. Lysogenic prokaryotes were observed at proportions of (a)

less than 10% in temperate lakes (Colombet et al., 2006) and coastal seawaters (Weinbauer and Suttle, 1999); (b) from 10% to 50% in offshore waters (Jiang and Paul, 1996; Bongiorni et al., 2005), and (c) from 50% to 100% in estuaries (Cochran and Paul, 1998), Antarctic saline lakes (Laybourn-Parry et al., 2007) and deep marine waters (Weinbauer et al., 2003). However, this variation can also be related to the method used to determine the lisogenic fraction of prokaryotes, since two methods can be used: (1) the decrease in prokaryotes counts in the presence of inductants (mortality method), and (2) dividing the number of viruses increased by an average burst size (average burst size method).

In the first methods, according to Williamson et al (2002), the lisogenic fraction (LF) can be determined by (Bc-Bi)/Bc*100, where Bc and Bi are the number of prokaryotes enumerated in the control and induced samples after incubation, usually 18 hours, respectively with the inductant. In this method it is assumed that the increase in viral numbers and the decrease in prokaryotes numbers in the induced samples were caused solely by lysogenic induction. It is no taken into account mortality by grazing and senescent cell death. This method ignored also mortality caused by toxicity of the inducing agent, overestimating lisogeny (Jiang and Paul (1994, 1996). In the second method, according (Williamson et al., 2002) the lisogenic fraction is determined by the formula LF= [(Vi-Vc)/Bz]/Bc*100 where Bc is the number of prokaryotes enumerated in the control sample after incubation, usually 18 hours and the Bz is the burst size the Vi is the number of viruses enumerated in the induced sample at 18h and the Vc is the number of viruses enumerated in the control sample. This method is more accurate because mitomycin C is not toxic for viruses, but with this method, prokaryotes containing few viral particles (≤ 6) are not included in the determination (Jiang and Paul,. 1996). With this method aggregated viruses are also counted as bursting prokaryotic cells and phage particles attached to prokaryotes surface may also be counted as mature particles inside a cell (Jiang and Paul, 996). High burst sizes may lead to the underestimation of the presence of lisogeny in prokaryotic communities (Jiang and Paul, 1996). This method is the most used in the literature because it gives more realistic values of the lisogenic fraction.

1.7 Lisogeny at the Surface Microlayer

The Surface microlayer (SML) (top 1 to 1000 μm of the water column), represents the interface between the atmosphere and the hydrosphere, and is characterized by physical, chemical and ecological properties distinct from the ones at underlying waters (UW) (Hardy, 1997). For prokaryotes, the SML can be an extreme environment, due to exposure to intense solar radiation in the ultraviolet and visible spectra, high concentrations of heavy metals and organic pollutants, temperature fluctuations and salinity changes. However, the SML also offers some advantages to microbial life, including high concentrations of organic and inorganic nutrients (Lion and Leckie, 1981). Prokaryotes abundance at the surface microlayer has been shown to be 10^3 to 10^5 higher comparatively to underlying waters (UW) (Bezdek and Carlucci, 1972). It has been also shown that lisogenic prokaryotes are more abundance at the SML, than at the UW (Tapper and Hicks, 1998; Bettarel et al, 2006). The frequent explanation has been the higher diversity of prokaryotes of the neuston that results in a larger diversity of potential host cells available to temperate viruses (Fehon and Oliver 1979; Carlucci et al. 1985; Hermansson et al. 1987). However, the percentage of viruses in the surface microlayer that retains their infectivity while being continuously exposed to sunlight is unknown.

1.8 Drawbacks of bacterial and viral determination by the epifluorescence microscopy method

The use of reliable methods for determination of bacterial abundance and biomass is an essential criterion for establishing the roles of bacteria in biogeochemical cycles and food chains. In addition, it is also important for understanding the dynamics of bacterial populations in natural systems.

The available methods for determining the bacterial abundance in aquatic environments are transmission electron microscope (TEM), epifluorescence microscopy and flow cytometry. The most widely used method is epifluorescence microscopy. In this method, bacteria are concentrated on 0.2 μm membranes, stained with fluorochromes and counted under an epifluorescence microscope (Buesing, 2005).

It is crucial to use fluorochromes specific to nucleic acids, in order to facilitate the differentiation between bacteria and other particles. This is particularly important when the samples are rich in organic matter. Acridine Orange (AO) and 4 ', 6-diamidino-2-phenylindole (DAPI), which stain only dsDNA, have traditionally been used for estimating the number of bacteria. However, in the last years, a variety of other dyes that bind to DNA and RNA have become commercially available. These include SYBR Green I and II, YOYO-1, YO-PRO-1, SYTO, and PicoGreen. The specificity and intensity of these dyes is much greater than that of DAPI and AO, facilitating the recognition and quantification of bacteria with greater accuracy (Mosier-Boss 2003) (Table 2)..

Epifluorescence microscopy is also the most widely method used to estimate viral abundance in aquatic systems. Comparatively to electronic microscopy, this method is faster, less expensive and more affordable, allowing the processing of a great number of samples and, consequently, to obtain more statistically accurate data. Epifluorescence microscopy is about seven times more accurate than TEM for counting viruses (Danovaro et al, 2001). The most frequently used stains to count viruses are DAPI, YOPRO-1, SYBRGreen and SYBRGold (Weinbauer, 2004). Contrarily to the other fluorochromes, DAPI stains only dsDNA viruses. Although in the marine environment dsDNA viruses are the most abundant, ssDNA, dsRNA and ssRNA viruses are also find in the aquatic systems and, consequently, are not covered by the DAPI staining.

As bacterial and viral abundance are determined by epifluorescence microscopy and the differentiation between bacteria and viruses is based on particle size, some bacteria can be counted as virus, namely in marine waters where bacteria are small (Almeida et al, 2001). Consequently, the number of viruses could be overestimated and the number of bacteria can be underestimated.

Table 2 Properties of the nucleic acid stains. Based in Haugland,RP.(1996) and Molecular Probes, BioProbes (1997)

Stains	Binding properties [a]
Dapi	Semi-permeant; AT selective; binds to dsDNA
Sybr gold	Permeant; binds to RNA, ssDNA, and dsDNA
Ethidium bromite	Impermeant; binds to RNA, ssRNA, dsDNA, tsDNA
YOPO-1	Impermeant; binds to ssDNA and dsDNA

a) ss 5 single stranded, ds 5 double stranded, ts 5 triple stranded; AT 5 adenine-thymine

2 Thesis outline

The importance of lisogeny as an alternative to lytic infection in natural populations of marine bacteria is poorly understood Williamson et al., 2002).

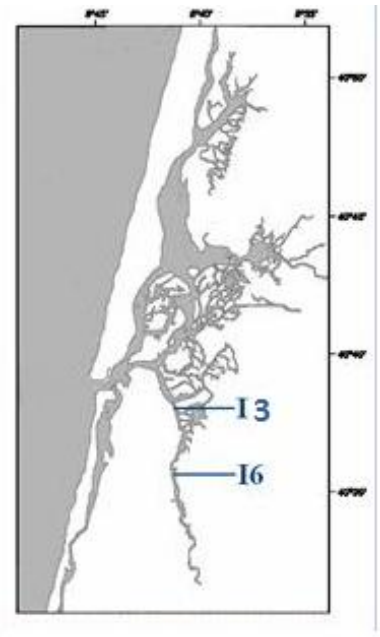
Wilcox and Fuhrman (1994) and Weinbauer and Suttle (1996) reported that the majority of viruses found in the marine environment are lytic and that lysogenic bacteriophages are quantitatively insignificant in coastal waters. Jiang and Paul (1994), however, showed that 43% of the bacteria isolated from various marine environments were lysogenic, as determined by prophage induction with mitomycin C.

With this work we tried to see if there is a significant quantitatively lisogenic bacteria in an estuarine environment, how bacterial community is affected and we tried to see variation between the two layers, SML and UW. Also in this work, the error associated with bacteria/viruses quantification by epifluorescence microscopy was determinate.

3. Material and Methods

3.1 Sampling Site

Ria de Aveiro is a coastal lagoon that stretches for 50 km along the coast of Portugal from Mira to Ovar and is separated from the sea by a sandy barrier. It exchanges with the sea a volume of water of 89 Mm^3 in tides with 1 to 3 m of amplitude (Silva, 1994) and receives freshwater from several rivers, with an average water input of 1.8 Mm^3 during a tidal cycle (Barrosa, 1985). The Ria has a complex topography, with several channels spreading from the mouth towards the different streams, forming a complex and dynamic estuarine system (Barroso, 2000)



3.2 Sampling

Water samples from the surface microlayer (SML) and the underlying water (UW) were collected in the marine zone (I3) and in the brackish water zone (I6) of Ria de Aveiro (Fig.3) in November 2008, March 2009, May 2009 and September 2009, during low tide. SML samples were collected with a 0.25 m wide, 0.35 m long Plexiglas plate (Harvey and Burzell, 1972), which roughly removes the upper 60–100 µm of the water column. Before sampling, the Plexiglas plate was cleaned with ethanol, rinsed with sterile distilled water and finally with water from the sampling site. The water adhering to the plate during immersion was removed from both sides by introducing the plate between two Teflon sheets and collecting the water into a sterilized glass bottle. Samples from UW were collected directly into 0.5 L sterile glass bottles from the depth of 20 cm. Triplicate sub-samples were analyzed for bacterial and viral parameters. Temperature and salinity were measured in the field with a WTW LF 196 Conductivity Meter (Wissenschaftlich Technische Werkstatten, Weilheim, Germany).

3.3 Experiments of lysogenic bacteria induction

The percentage of lysogenic bacteria was estimated after induction with the chemical agent mitomycin C. Three sub- samples of 20 mL that were collected from the SML and UW. Three sub- samples were treated with mitomycin C (1μg/mL, Sigma Chemical Co., St. Louis, Mo.) and the others were left untreated (control). Samples were well mixed and incubated in the dark for 18h, at room temperature.

In order to determine the percentage of lysogenic bacteria; the total number of bacteria and of viruses was determinated. The effects of lisogeny on the cultivable bacteria, and on the bacterial productivity were also studied.

3.3.1Bacterial and Virus Direct Counts

For bacterial abundance determination, samples (1 mL) were filtered through 0.2 μm pore-size polycarbonate membrane filter (Millipore) and stained with 400 μl of 2X SYBR Gold (Invitrogen) for 15 min. At least 20 fields in each duplicate of each sub-sample were counted per filter in a Leitz Laborlux K epifluorescence microscope with a 50 W mercury lamp equipped with a blue BP 450–490 exciter filter and LP 515 barrier filter.

Samples for virus enumeration (250 μl) were filtered through 0.2 μm pore-size polycarbonate membrane filter and the filtrated samples were filtered by 0, 02 μm pore size filters (Whatman; Anodisc, 25 mm diameter). Viral particles were stained on the filters for 15 min using 2x SYBR-Gold (Molecular Probes) and enumerated by epifluorescence microscopy. At least 200 viral particles were counted per filter for each duplicate of each sub-sample.

3.3.2 Cultivable bacteria

The abundance of inducible cultivable bacteria was determined by plating in PCA medium of treated and untreated samples. The plaques were incubated in the dark at 25ºC for 5 days, after which the number of colony forming units (CFU) per milliliter was determined

3.3.3 Bacterial Biomass Productivity

Bacterial biomass productivity (BBP) was estimated by the incorporation of ^{3}H leucine into bacterial protein, using 1.5 mL triplicate of each sub-samples plus a control fixed with TCA (2% final concentration) The samples were incubated in the presence of a saturating concentration (30 nM) of 3H-leucine (Amersham, specific activity of 63.0 Ci.nmol^{-1}) for 1 hour in dark at *in situ* temperature. After incubation, reactions were stopped with 2% TCA and the tubes were centrifuged from 10 min at 14,000 rpm. The pellet was washed two times with 1 mL of ice-cold 5% TCA and then with 1 ml of cold ethanol. After overnight drying of the pellet, 1 ml of scintillation cocktail UniverSol (ICN Biomedicals, USA) was added. Radioactivity was measured after a period of 3 days in a Beckman LS 6000 IC liquid scintillation. The conversion of leucine incorporation rates to carbon units was accomplished according to Simon and Azam (1989).

3.3.4 Calculations of induced lysogenic fraction and burst size

The amount of induced prophage was calculated as $V_I - V_C$, where V_I is the number of viruses enumerated in the induced sample at 18 h and V_C is the number of viruses enumerated in the control sample. The lysogenic fraction (LF) of the bacterial population was determined by two methods. The first, termed the burst size method, was calculated using the following equation: LF = $[(V_I - V_C)/B_Z]/B_C \cdot 100$, where B_C is the number of bacteria enumerated in the control sample at 18 h and B_Z is the burst size. An average value of burst size of 30, derived from transmission electron microscopy observation of Tampa Bay samples *(*Jiang and Paul, 1996) was used. The second method for calculation of the lysogenic fraction used the following equation: LF = $(B_C - B_I)/B_C \cdot 100$, where B_C and B_I are the number of bacteria enumerated in the control and induced samples at 18 h, respectively. This was corrected by subtracting the average mortality caused by mitomycin C in induced samples. Induced burst size (BZ_I) was calculated using the following equation: $BZ_I = (V_I - V_C)/ (B_C - B_I)$.

3.3.5 DGGE (Denaturing Gradient Gel Electrophoresis)

For the characterization of the bacterial community structure after the 18h incubation with mitomycin C. A 200 mL volume of test and control samples were filtered through 0.2 µm polycarbonate filters (Poretics Products Livermore, USA). Collected cells were resuspended in 2 mL of TE buffer [10mM Tris HCl, 1mM ethylenediamine tetraacetic acid (EDTA), pH 8.0] and centrifuged. Lysozyme solution (2 mg mL^{-1}) was added to induce cell lysis, followed by incubation at 37ºC for 1 h according to the procedure described by Henriques et al (2004). DNA extraction was performed using the Genomic DNA Purification kit by MBI Fermentas (Vilnius, Lithuania). DNA was resuspended in TE buffer and stored at –20ºC. The yield and quality of DNA were checked by electrophoresis on a 0.8% (w/v) agarose gel. PCR amplification of an approximately 400 bp 16S rDNA fragment (V6-V8) was performed using the primer set F968GC and R1401 (Nubel *et al*., 1996). The reaction was carried in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) from MIDSCI. The PCR reaction mixture (25 µL) contained, approximately 50 to 100 ng of extracted DNA; 1x PCR buffer (PCR buffer without $MgCl_2$: PCR buffer with KCl_2, 1:1); 2.75 Mm $MgCl_2$; 0.2mM of each nucleotide; 0.1µM of each primer; and 1 U of Taq Polymerase (all reagents purchased from MBI Fermentas, Vilnius, Lithuania). Acetamide (50%, 0.5µl) was also added to the reaction mixture. The amplification protocol included a 4 minute initial denaturation at 94ºC, 34 cycles of 95ºC for 1 minute, 53ºC for 1 minute and 72ºC for 1 minute and 30 seconds, and a final extension for 7 minutes at 72ºC. After amplification, 5 µL of the PCR product was subjected to electrophoresis on a 1.5% (w/v) agarose gel stained with ethidium bromide (0.01% v/v). DGGE was performed with the Decode System (CBS Scientific Company, Del Mar, CA, USA). Approximately equal amounts of PCR products were loaded onto 6-9% polyacrylamide gel in 1x TAE buffer (20

mM Tris, 10 nM acetate, 0.5 mM EDTA, pH 7.4). The 6-9% polyacrylamide gel (bisacrylamide: acrylamide = 37.5:1) was made with a denaturing gradient ranging from 32 to 60% (100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at 60ºC for 16 hours at 150 V. Following electrophoresis, the gels were silver stained. After fixation with 0.1% (v/v) ethanol, 0.005% acetic acid for fixation, 0.3 g silver nitrate for 20 min, freshly prepared developing solution containing 0.003% (v/v) formaldehyde, 0.33% NaOH (9%) was added. The development was stopped using a0.75% sodium carbonate solution (Heuer et al., 2001). Gel images were acquired using a Molecular Image FX apparatus (Bio-Rad). The impact of the mitomycin C effect on the diversity of bacterial community was assessed by determination of the number of bands in DGGE images in the samples and controls after the incubation of 18h.

3.5.1 Statistical Analyses

The DGGE gels were scanned and the digitalized DGGE profiles were analyzed with the software package Gelcompar 4.0 program (Applied Maths) as described by Smalla et al. (2001). Bands were searched in the DGGE profiles by using the sets for minimal profiling and minimal area at 5% and 0.5%, respectively. Positioning and quantification of bands present in each lane was carried out by setting tolerance and optimization at 8 points, i.e. 0.8%. The band positions and their corresponding intensities (surface) from each water sample treatment were exported to Excel and the band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane.

Based on Bray–Curtis similarities, multivariate analyses of DGGE profiles were performed using analysis of similarities (ANOSIM) and nonmetric multidimensional scaling (MDS) with the PRIMER 5 software package (Primer-E Ltd, Plymouth UK). The ANOSIM was used to test whether there are separation (R = 1) or not (R = 0) between bacterial communities from different groups of samples. The null hypothesis is that there are no differences in the composition of bacterial communities from the water samples from control and the water samples with mitomycin C after 18 h of incubation. In general higher R values than 0.25 will indicate greater variation between groups. Differences in the bacterial community structure were assessed graphically using multidimensional scaling (MDS) (Yannarell et al, 2005).

3.3.6 Experiments to determinate prokaryotes counted like virus

To determinate the percentage of prokaryotes that are counted like viral particles samples were collected from station I3 and I6 and it was used Fluorescence in situ Hybridization (FISH) with Cy3-labeled oligonucleotide probes (Amann et al. 1990). Samples (1 mL) were filtered through 0.2 µm pore-size membranes and the filtrate was further filtered through 0.02 µm pore-size membranes. Samples were fixed with 2% paraformaldehyde for 30 min and rinsed with distilled water and the filters reserved in the dark until hybridization.

Domain and group specific probes were used for bacteria Eub338 (Amann et al. 1990) and for archaea Arc 915 (Stahl et al., 1988). For each probe triplicate filter pieces were placed on Parafilm-covered glass slides and overlaid with 30 µL hybridization solution with 2.5 ng/µL of probe (final concentration). The hybridization solution contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% SDS, and the optimum concentration of formamide (Eilers et al. 2000; Zarda et al. 1997). Filters were incubated in a hybridization oven at 42°C for 90 min. After hybridization filters were washed for 20 min at 48°C in wash solution (20 mM Tris HCl pH 7.4, 5 mM ethylenediamine tetraacetic acid, 0.01% SDS, and the appropriate concentration of NaCl) (Eilers et al. 2000; Zarda et al. 1997). Rinsed and dried filter pieces were mounted with 4:1 Citifluor: Vectashield containing 2 µg/mL 4', 6-diamidino- 2-phenylindole (DAPI). Samples were analyzed in a Leitz Laborlux K microscope.

Counts were made in a Leica epifluorescence microscope equipped with filter for Cy3 (Cy3 - Y3, Leica) and a filter for DAPI (DAPI - A, Leica). For each sample observed three replicates, were counted 10 random optical fields in each replica. In each optical field proceeded to count the cells stained with DAPI and labeled cell probes.

Results

4.1 Water Properties

Water temperature was higher during the warmer season, reaching values of 22ºC in the brackish water zone. The lowest values were observed in November. Salinity was higher in November in both sampling stations varying between 20.3 and 30.9 (Table 3).

Table 3 Physic-chemical characterization of water samples collected in the Ria de Aveiro, stations I3 and I6 for SML and UW.

Date of Sampling	I3	SML	UW	I6	SML	UW
27-11-2008	Salinity	29,8	29,5	Salinity	30,9	31,2
	Temperature		10,8ºC	Temperature		9ºC
26-03-2009	Salinity	23,8	26,6	Salinity	23,1	22,9
	Temperature		18ºC	Temperature		17,5ºC
08-05-2009	Salinity	29,9	31	Salinity	20,3	26,7
	Temperature		18,8ºC	Temperature		19,8ºC
22-09-2009	Salinity	27,8	29,0	Salinity	24,8	26,9
	Temperature		18,4ºC	Temperature		18,7ºC

4.2 Variation of total and cultivable bacteria during the lisogenic induction experiments

Total bacterial number was highest in November for station I3 (9, 14E+06) and I6, (9, 03E+06) and lowest values were observed in March (Fig.4). Total bacterial abundance was always higher in the SML relatively to UW (Fig. 4).

The addition of mitomycin resulted in a decrease in total bacterial abundance. In station I3, the decrease ranged from 35% to 71 % in the SML and from 38 % to 59% in the UW. In station I6, the addition of mitomycin resulted in a decrease of 49 % to 71% in SML and of 43 % to 71% in the UW.

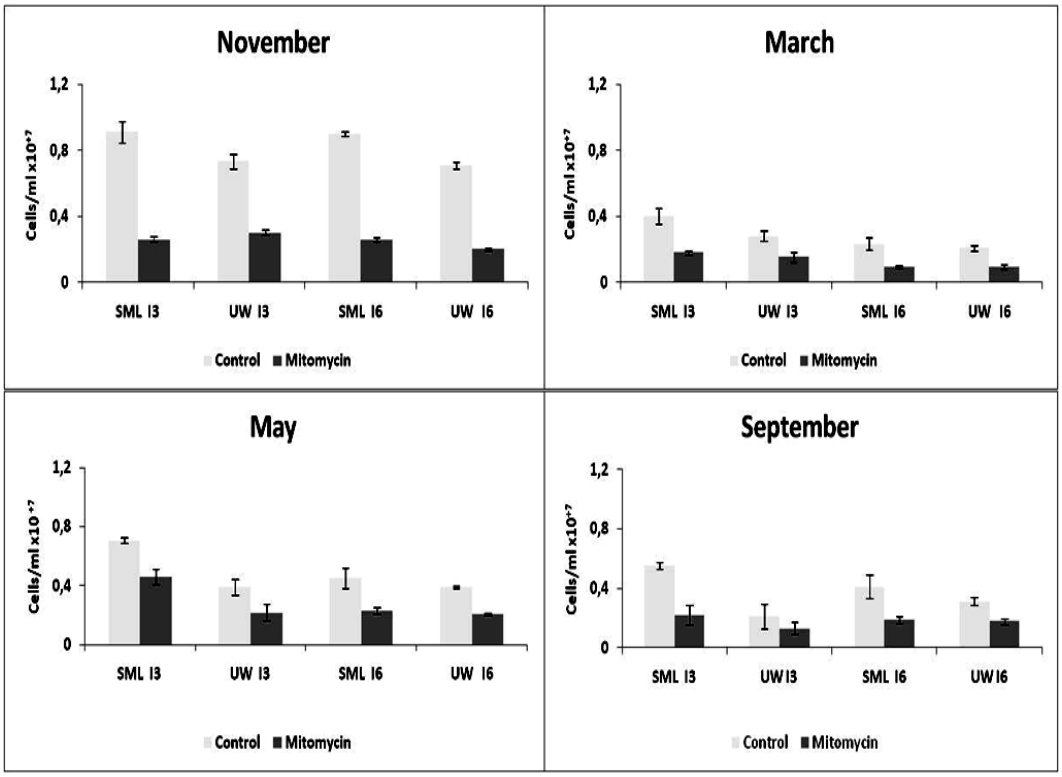


Figure 4 Variation of the total number of prokaryotes in SML and UW of I3 and I6 sampling stations before and after the addiction of mitomycin C

The abundance of cultivable bacteria varied with the sampling date (Fig5). Cultivable bacteria were more abundant in November both in the marine and brackish water zone of the estuary., The lowest abundance in cultivable bacteria were observed in May in the marine station (1,08E+03) and in September for the brackish water section (1,90E+02). Cultivable bacteria abundance was always higher in the SML for both zones. The station I3 presented, in general the highest values.

The addition of mitomycin C resulted in a decrease in the abundance of cultivable bacteria At SML and at UW. In the marine r station I3 mitomycin-induced decrease of the cultivable bacteria of 16% to 90% in the SML and of 16% to 64% in the UW. The highest decrease in cultivable bacteria abundance in mitomycin-treated samples was observed in March, for both water layers. In the brackish water section I6, the addition of mitomycin resulted in decreases of cultivable bacteria abundance of 22 % to 77% at the SML and 12 % to 96% in the UW.

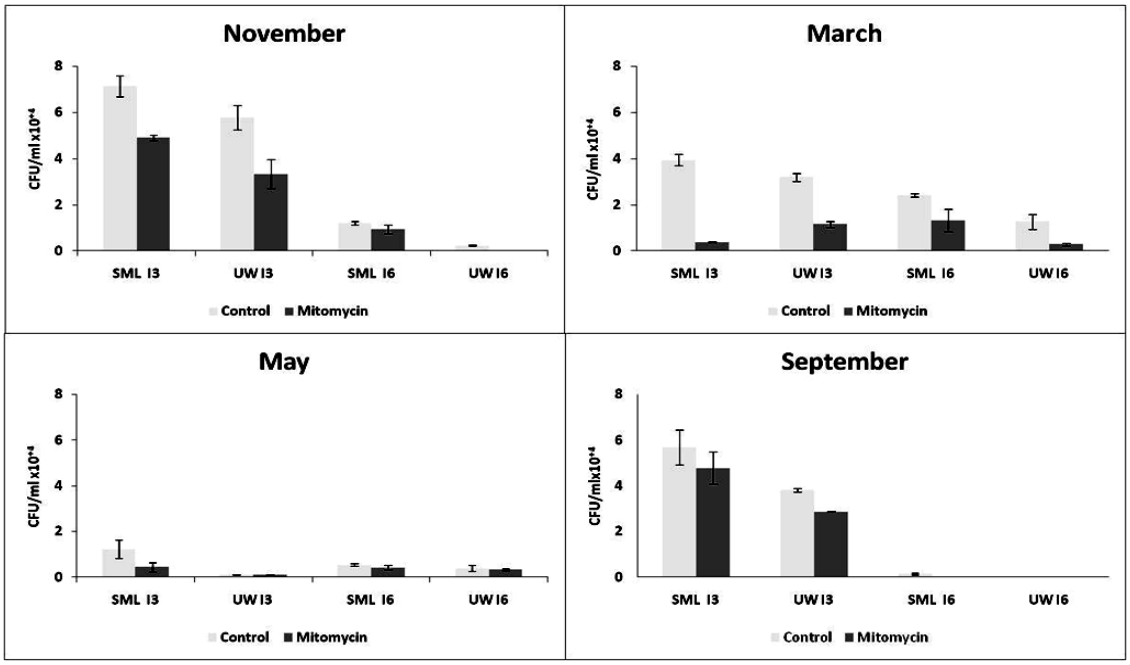


Figure 5 Variation of cultivable bacteria in SML and UW of I3 and I6 sampling stations before and after the addiction of mitomycin C

4.3 Variation of Bacterial Biomass Productivity (BBP) ring the lisogenic induction experiments

The highest values of BBP occurred in September for both the marine (75 μg C L^{-1} $h^{-1)}$ and brackish water (45 μg C L^{-1} h^{-1}) stations. In station I3, BBP ranged from 18 μg C L^{-1} h^{-1} to 74 μg C L^{-1} h^{-1} in the SML and 18 μg C L^{-1} h^{-1} to 64 μg C L^{-1} h^{-1} in the UW. In station I6, the values of BBP varied from 12 to 45 in the SML and 11 μg C L^{-1} h^{-1} to 28 μg C L^{-1} h^{-1} in the UW (Fig.6).

The addition of mitomycin C resulted in a decrease in BBP. In station I3, mitomycin-treated samples showed a 21% to 84 % decrease in the SML while in the UW the decrease observed ranged from 62% to 85%. In station I6, mitomycin induced a decrease in BBP ranging from 69 to 91% in SML and from 35 % to 90% in the UW.

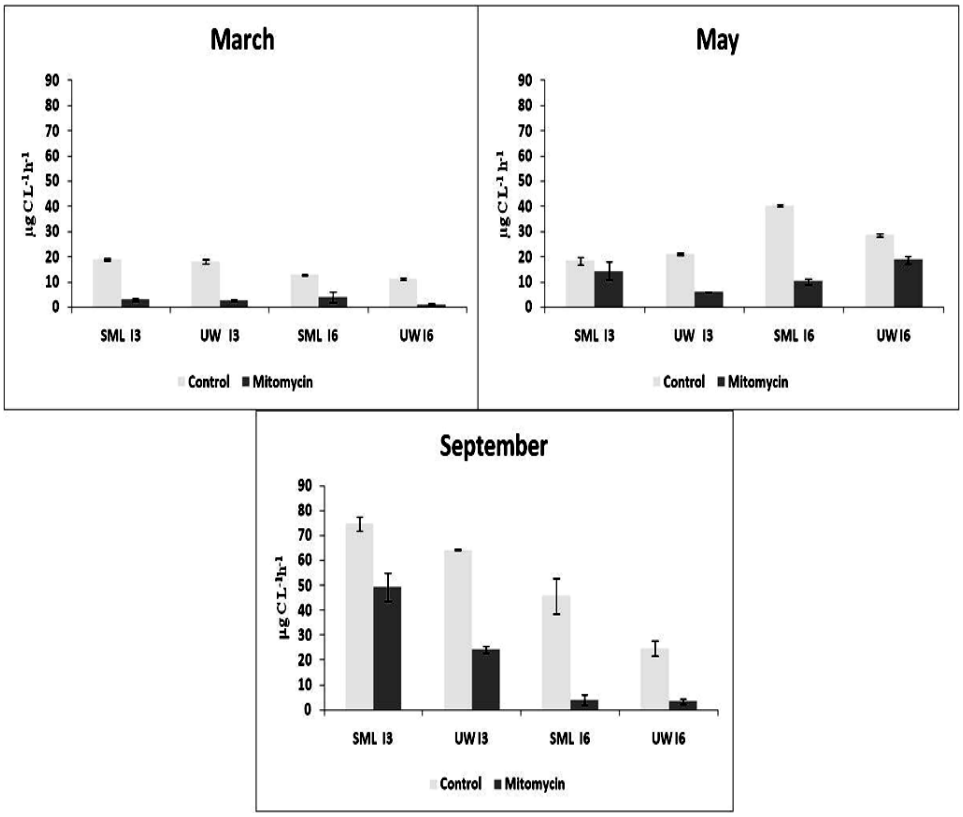


Figure 6 Variation of the Bacterial Biomass Productivity in SML and UW of I3 and I6 sampling stations before and after the addiction of mitomycin C

4.4 Variation of total viral number and of burst size during the lysogenic induction experiments

The abundance of viral particles was highest in November and lowest in March varying between 3, 5 x 10^6 and 3, 3 x 10^7(Fig.7) . The viral abundance was higher in the SML in all the sampling dates. The addition of mitomycin C resulted in an increase in viral particles abundance, ranging from 24 % to 37% in the SML and 16% to 130% in the UW. In station I6, mitomycin C induced increases of viral particles varied between 9% to 69% in SML and between 30% and 47 % in the UW.

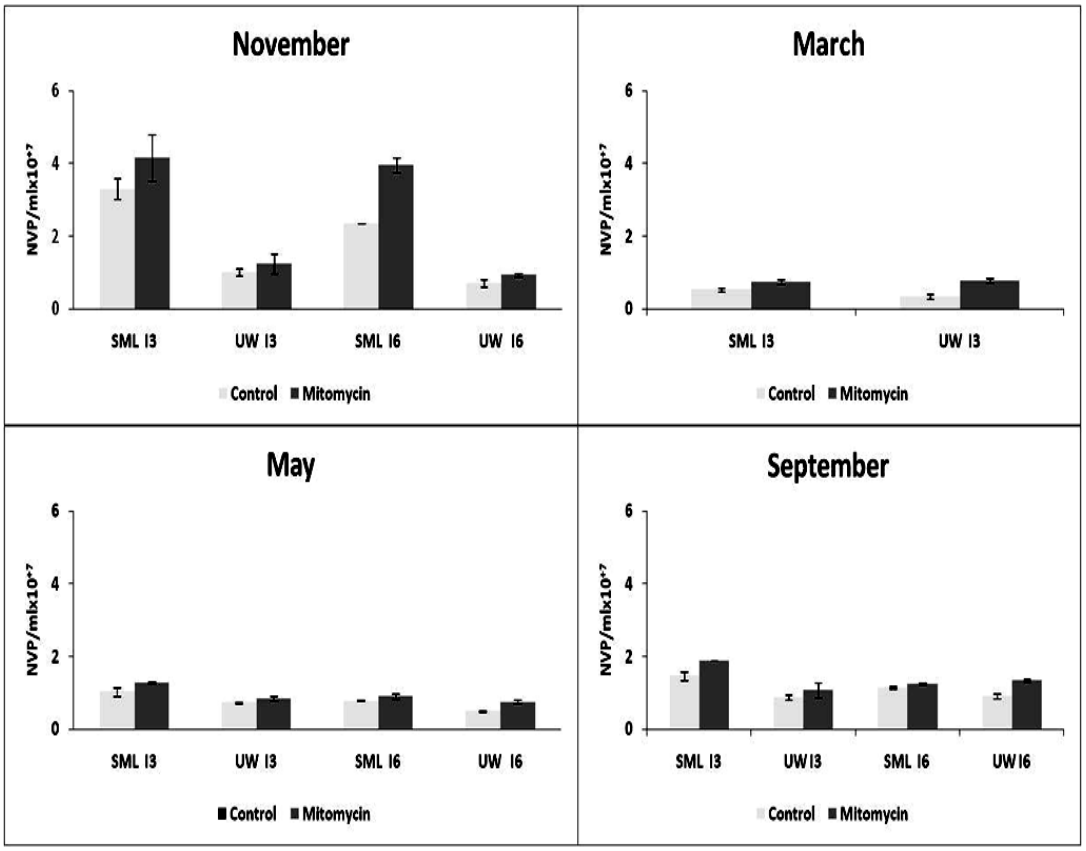


Figure 7 Variation of the total number of viral particles in SML and UW of I3 and I6 sampling stations before and after the addiction of mitomycin C

Using the formula of the average burst size the relative abundance of lysogenic bacteria varied between 1.2 % and 3.1% in the SML and from 1.0% and 5.3% in the UW at the marine water station. In the brackish water section, the percentage of lysogenic bacteria varied from 0.9% to 6.0 % in the SML and from 1.0% to 4.7 % in the UW (Table.4).

Using the mortality formula was observed that the lisogenic fraction was higher varying from 34.8% to 71.3% in the SML and from 38.0% to 58.7% in the UW at the marine zone. In the brackish water section, the percentage of lysogenic bacteria varied from 48.7% to 71.1 % in the SML and from42.6% to 71.3 % in the UW (Table 4).

When we used the formula of the average burst size was observed that the higher percentage of lisogenic fraction in the marine zone varied between layers. In the brackish water station, the induction of lisogeny was in general higher in the UW (except November). With the prokaryotes mortality formula, it was observed that in the marine

station, in general, the induction of lisogeny was highest at the SML (except in May). In the brackish water station, the induction of lisogeny was also in general higher in the SML (except in November).

Table 4 Induction of lisogeny in water samples collected in the Ria de Aveiro by mitomycin C, quantified by the average burst size 30 and the mortality formula.

Month	Samples			PVN ml^{-1}	TBNml^{-1}	Mortality	Average Burst Size (30)
November	I3	SML	Control	3,31E+07	9,14E+06	71,3	3,1
			Mitomycin C	4,16E+07	2,62E+06		
		UW	Control	1,02E+07	7,35E+06	58,7	1,1
			Mitomycin C	1,25E+07	3,03E+06		
	I6	SML	Control	2,35E+07	9,03E+06	71,1	6,0
			Mitomicina C	3,97E+07	2,61E+06		
		UW	Control	7,27E+06	7,11E+06	71,3	1,0
			Mitomycin C	9,48E+06	2,04E+06		
March	I3	SML	Control	5,42E+06	4,02E+06	54,3	1,7
			Mitomycin C	7,45E+06	1,84E+06		
		UW	Control	3,45E+06	2,82E+06	44,9	5,3
			Mitomycin C	7,93E+06	1,55E+06		
	I6	SML	Control	ND	2,36E+06	ND	ND
			Mitomycin C	ND	9,64E+05		
		UW	Control	ND	2,10E+06	ND	ND
			Mitomycin C	ND	9,77E+05		
May	I3	SML	Control	1,04E+07	7,07E+06	34,8	1,2
			Mitomycin C	1,29E+07	4,61E+06		
		UW	Control	7,39E+06	3,89E+06	44,5	1,0
			Mitomycin C	8,57E+06	2,16E+06		
	I6	SML	Control	7,99E+06	4,49E+06	48,7	0,9
			Mitomycin C	9,18E+06	2,31E+06		
		UW	Control	5,14E+06	3,92E+06	47,2	2,0
			Mitomycin C	7,54E+06	2,07E+06		
September	I3	SML	Control	1,48E+07	5,52E+06	60,5	2,5
			Mitomycin	1,89E+07	2,18E+06		
		UW	Control	8,87E+06	2,11E+06	38,0	3,4
			Mitomycin	1,10E+07	1,31E+06		
	I6	SML	Control	1,15E+07	4,09E+06	54,5	0,9
			Mitomycin	1,21E+07	1,86E+06		
		UW	Control	9,18E+06	3,10E+06	42,6	4,7
			Mitomycin	1,35E+07	1,78E+06		

TBN, total prokaryotic number; PVN , total viral number; ND- Not determined.

4.5 Bacterial diversity in underlying water after 18h of incubation with and without mitomicina.

The DGGE profiles resulting from the separation of fragments of 16s rDNA genes amplified by PCR are shown in the figures 8 and 9.

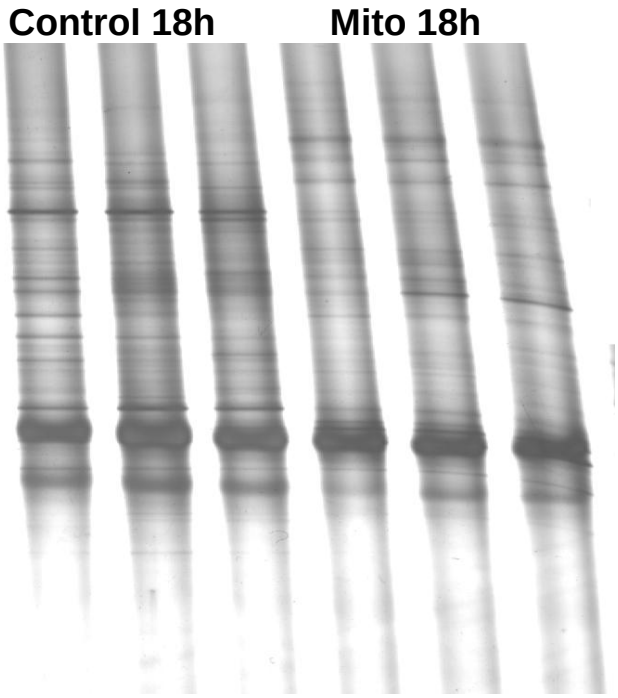


Figure 8. DGGE profile of the water samples of station I3 without mitomycin C (control) and with mitomycin C after 18h of incubation.

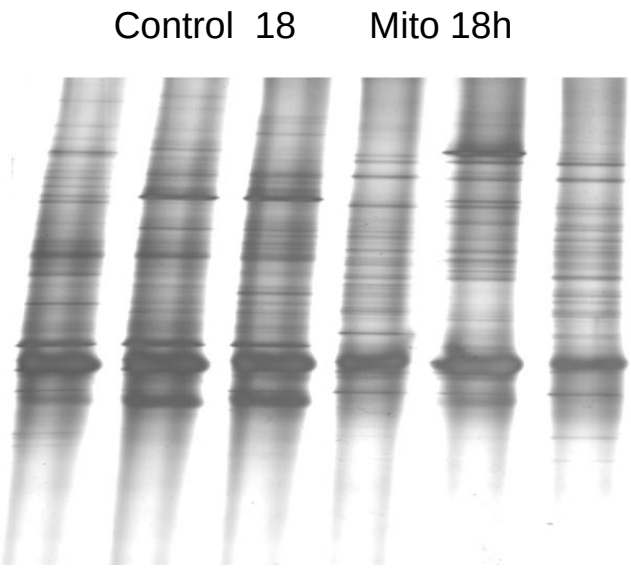


Figure 9. DGGE profile of the water samples of station I6 without mitomycin C (control) and with mitomycin C after 18h of incubation.

Analyzing the two DGGE profiles of both stations it is observed that in the samples with mitomycin C some bands disappeared when we compare with the control but others that are not observed in the DGGE profile of the control appeared in the DGGE profile of the samples with mitomycin C.

Using the multidimensional scaling (MDS) it is observed that the samples with the mitomycin C and without mitomycin are well separated in both stations (Figures 10 and 11).

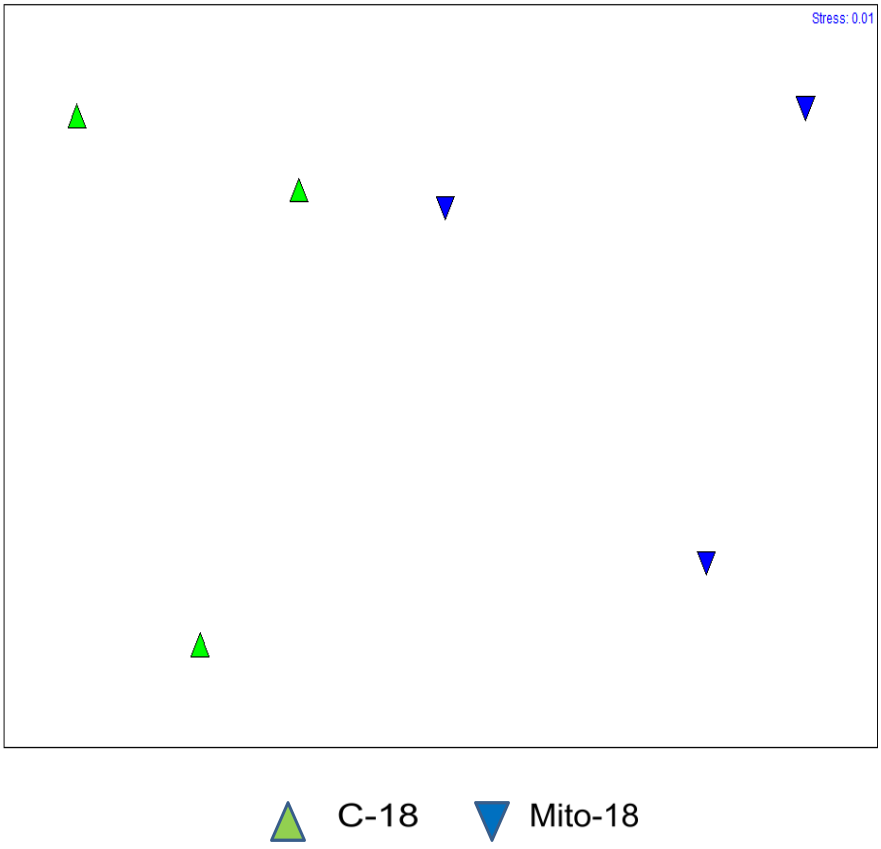


Figure 10. MDS analysis in station I3.

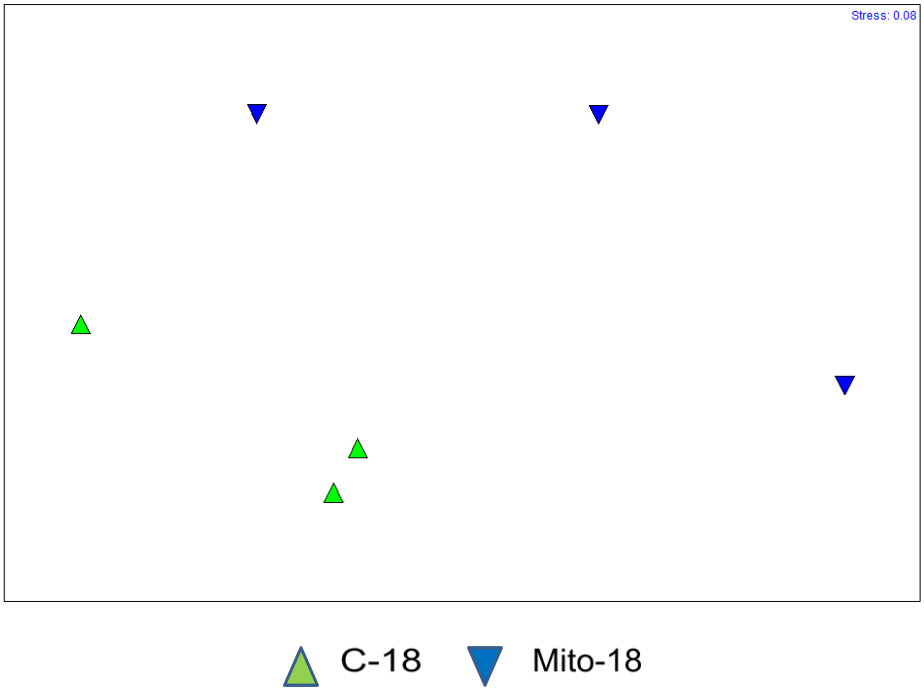


Figure 11. MDS analyses in station I6.

Using the ANOSIM, it was observed that there is a significant difference between the samples with mitomycin and without mitomycin C. In the station I3 the R is 0, 63 and in the station I6 the R is 0, 37.

4.6 Fraction of prokaryotes counted as viruses

When the samples were filtered by membranes of 0,2 µm it was clear the dominance of Eubacteria in comparison to Archaea. The relative abundance of Eubacteria was 68 % in station I3 and 59 % in station I6, while the relative abundance of Archaea was 8% in I3 and 11 % in I6 (Fig.12) (Table 5 and 6). When samples were filtered trough 0,02 µm pore size it was observed that the percentage of viral particles increased and the percentage of prokaryotes decreased. The abundance of Eubacteria ranged from 9% in I6 to 20% in I3, while the relative abundance of Archaea varied between 7 % in I3 and 5 % in I6 (Fig.12) (Table 5 and 6).

Table 5 Percentage of Prokaryotes and viral particles filtered by 0, 2 µ m

I3	Eubacteria	68%
	Archaea	8%
	Viral particles	24%
I6	Eubacteria	59%
	Archaea	11%
	Viral particles	30%

Table 6 Percentage of Prokaryotes and viral particles filtered by 0, 02µm

I3	Eubacteria	20%
	Archaea	7%
	Viral particles	73%
I6	Eubacteria	9%
	Archaea	5%
	Viral particles	86%

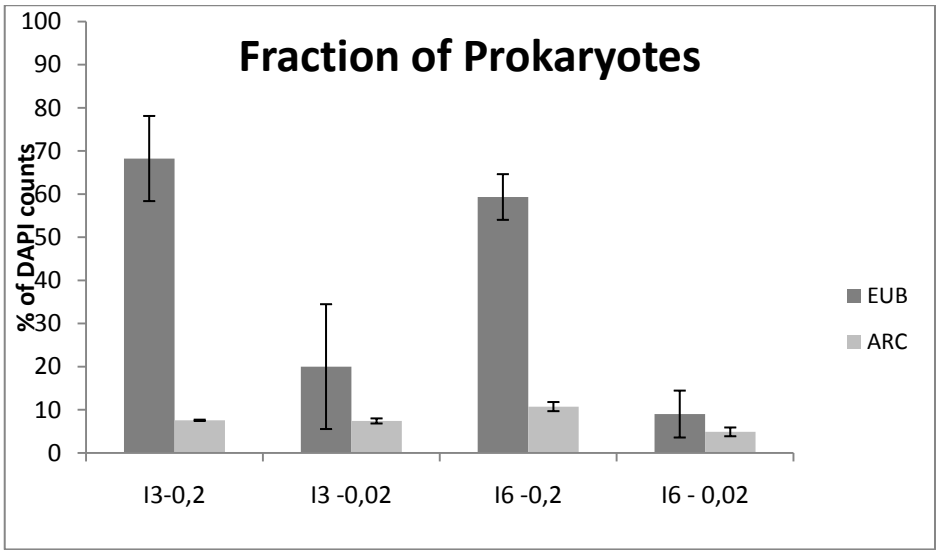


Figure 12 Relative abundance of Prokaryotes filtered by 0,2µm and 0, 02µm

5. Discussion

The study of ecological significance and role of viruses in their natural habitats is a fast growing field of research. It is well known that viruses are the dominant biological component in aquatic systems, and are; at least, 10 fold more abundant than bacteria and archaea (Suttle. 2007). A significant proportion of isolated bacteria from the aquatic systems harbour viruses in their genome and some of them are polylysogenic, that is, have more than on viruses integrated in the genome (Appleyard 1954). The high number of lysogenic prokaryotes in the environment results from the co-evolution of viruses and prokaryotes and it has been shown that lisogeny may be a survival strategy for viruses in the marine environment by preventing the destruction of their hosts (Cochran and Paul, 1998). However, the importance of lisogeny as an alternative to lytic infection in natural populations of marine prokaryotes is poorly understood.

The results of this study show that in Ria de Aveiro, a small fraction of prokaryotes is lysogenic, increasing when the environmental conditions are adverse and that the induction of lisogeny can affect not only the prokaryotes abundance and activity but also the bacterial community structure.

In the estuarine system Ria de Aveiro, the lysogenic fraction of prokaryotes varied from 1.0 to 5.3 % in the marine zone and from 0.9% to 6.0% in the brackish water zone. These values are similar to those find in other estuarine environments, using the increase in viral number to determine lisogeny. Weinbauer and Suttle (1999) determined lisogenic fractions of 0.07% for 4.4% in average 1.5% and Cochran and Paul (1998) showed that in Tampa Bay the lisogenic fraction ranged from 0% to 37.3% an average of 6.9% prokaryotes are lisogenic. However, Jiang and Paul (1996) found that 1.5% to 38% average of 8.8 % of prokaryotes in Gulf of Mexico carry temperate phages and Williamson et al (2002) determined a lisogenic fraction of around 50% for Tampa Bay. Jiang and Paul (1994) found that 43% of heterotrophic bacteria isolated from estuarine environment contained inducible prophage. The variation observed for the percentage of lisogeny

detected in environment with similar characteristics can, in part, be due to the approach used to its determination.

Even when it is used the same method to determine lisogeny, decrease in prokaryotic counts or increase of the number of viruses, other variables influences the determination. For instance, when lisogeny is determined using the increase of the number of viruses, the way to determine the burst size is a factor of variation. In the majority of the lisogenic induction studies it is used an average burst size of 30 (Jiang and Paul, 1996) or 50 (Wommack and Colwell, 2000) but the burst size can be calculated for each case by counting the number of viruses infecting prokaryotes, using transmission electronic microscopy. Moreover, the number of viruses can be determined by epifluorescence microscopy or by transmission electronic microscopy and the accuracy and precision of these both methods is different (Weinbauer and Suttle, 1997). In this study (Table 4), it was determined that 0.9 to 6.0 % (average, 2.5 %) of the indigenous prokaryotes present in the Ria de Aveiro are lisogenic, as calculated by dividing the number of viruses increased by an averaged burst size of 30. The percentage ranged from 34.8 to 71.3% (Table 4) (average, 53 %) when prokaryotic mortality is used to determine the lisogenic fraction. Jiang and Paul (1994) and Cochran and Paul (1998) obtained also high percentages of lisogeny when they used the decrease in prokaryotic counts in the presence of inductants. The difference between the two methods can be due to the fact that the method based on prokaryotic mortality assumes that the increase in viral numbers and the decrease in prokaryotic numbers in the induced samples are caused solely by lisogenic induction. However, the toxic effects of mitomycin may be the reason for the high loss rates of prokaryotes. On the other hand, the low lisogenic prokaryotic fraction detected in this study relatively to other estuarine systems can be associated to the use at a standard burst size that can be not the correct one for this system.

Contrarily to the observed in others studies (Wilcox and Fuhrman, 1994; Weinbauer and Suttle. 1999), lisogeny was not less prevalent in the more nutrient-rich section of the estuarine system. The percentage of lisogeny was similar in both sampling stations, althouhg it has been demonstrated that in the brackish water sections nutrient concentration is double of that of the marine zone (Almeida et al, 2001). None of the

environmental parameters measured in this study explain the same percentage of lisogeny in both zones.

As lisogeny induction is affected by the environmental factors, it is ecologically important to study the lisogeny induction in the SML where prokaryotes are exposed to intense solar radiation, high temperature, salinity gradients, toxic organic substances and heavy metals (Liss and Duce, 1997), but where dissolved and particulate organic matter are enriched up to 1000 times compared to UW (Liss and Duce, 1997). Although prokaryotes in the SML are more stress exposed , they may also be protected within the organic matrix. With the exception of November, when SML showed higher values of lisogeny, the lysogenic bacteria were similar in both SML and UW or even higher in UW. These results are in accordance with those of Weinbauer et al (2003), but different from those obtained frequently in other environments, which showed a higher fraction of lisogeny in the SML (Tapper and Hicks, 1998; Bettarel et al., 2003 and Bettarel et al., 2008). In general, the enhanced abundance of lisogenic prokaryotes in the SML is explained by the high exposure of this compartment to UV radiation and by the high levels of pollutants observed in this layer (Cochran and Paul, 1998). However, as UV radiation is a natural inductor of lisogeny and the water samples were sampled during the day, the correct effect of the artificial inductor may not be detected. In fact, a study about the impact of artificial ultraviolet-B radiation (0.4 W m-2) on the abundance and activity of the bacterial community of SML and of UW in Ria de Aveiro (Santos et al, 2009) showed that, in general, bacterioneuston is more affected by UVR exposure than bacterioplankton. For a better quantification of the lisogenic bacterial fraction in SML, the water samples must be collected during the night or before sun rising. Actually, previous studies in Ria de Aveiro, indicate that the differences between SML and UW are highly variable during the day, pointing out to the importance of the sampling moment to comparative studies of neuston and plankton (Santos et al, 2009). This conclusion may be also applied to the determination of lisogeny in prokaryotes.

High frequency of induction events have been reported to occur in periods of low water temperature, rainfall, nutrient concentrations, primary productivity and bacterial productivity (Williamson et al., 2002). In Ria de Aveiro, the highest abundance of lisogenic

prokaryotes in the SML occurred in November (Table 4), for both sampling stations, when salinity was higher and water temperature was low. In the UW the highest value of lisogenic fraction at station I3 occurred in March when the bacterial production was low, but at station I6 the highest value occurred in September 4,7 % and none of the parameters studied seems to explain this high value in May.

The results suggest that lisogeny can be frequent for cultivable heterotrophic bacteria, affecting their density and activity. As for the total prokaryotic community the number of cultivable bacteria decreases after the addition of the mitomycin as seen in figure 5. However, the difference between the added and non added samples in November and in May was not as high for the cultivable fraction of bacteria as that observed for the total community (Fig.5). This different pattern of variation can be due to differences in the prokaryotic community composition. In fact, in Ria de Aveiro it has been observed a clear pattern of seasonal variation in SML and in UW (unpublished data). It is important to note, however, that the decrease in bacterial counts and activity can be also due to the toxicity of the mitomycin C. In fact, the difference between the reduction of prokaryotic density and the increase of viral density was not proportional for both water layer and for both sampling stations during the sampling period. This suggests that the decrease in prokaryotic abundance and activity can not be due only to viral lysis, but also due to the toxicity of the inducing agent. Really, when prokaryotic mortality is used to determine the lisogenic fraction (this method assumed that the increase in viral numbers and the decrease in prokaryotic numbers in the induced samples were caused solely by lisogenic induction) the percentage of lisogeny is much higher.

Viruses have a restricted range of host cells, often a single species or even a single strain of prokaryotes. Consequently, infection by a particular virus does not act on the total bacterial assemblage, but rather on specific subpopulations. Viruses, therefore, have the potential to significantly influence the species composition of microbial communities by selective elimination of specific subpopulations, and viruses may contribute to maintaining a high microbial diversity by lysing numerically dominant strains/species. The results of this study confirm that in the aquatic environment viruses can control the diversity of the bacterial community. The bacterial community structure was significantly

altered after lisogenic induction. The DGGE profiles show that some groups of bacteria disappeared after induction of lisogeny but other bacterial groups, not detected in the controls, appeared after lisogeny induction. It is well know that some groups of bacteria that resist to viral infection benefit from organic matter released during viral infection of other bacterial groups (Weinbauer et al., 2003), which explain the intensification of some bands in the DGGE profiles after lisogeny induction. As lisogeny is a common occurrence among bacteria of the estuarine system Ria de Aveiro and the inducing of lisogeny is frequent in the marine environments, bacteriophages have an important role in controlling bacterial diversity. However, mitomycin C can be toxic for bacteria (Paul and Jiang, 1994) and, as a result, affect directly bacterial community structure. In fact, as mentioned above, the discrepancy between the reduction of prokaryotic density and activity and the increase of viruses after lisogeny induction may due to the presence of mitomycin C and, therefore, affect bacterial diversity (Fig. 10 and 11).

In order to evaluate the ecological significance and role of viruses in the aquatic environment a method that is simple, accurate, and suitable for routine environmental analysis is needed. The high counting efficiency, ease of preparation, modest equipment requirements, and the possibility of preparing specimens for long-term storage, make the epifluorescence microscopy an ideal method for routine environmental analysis. The results obtained with epifluorescence microscopy indicate that the concentration of viruses in natural waters are higher than previously recognized and imply that the TEM-based method significantly underestimates virus abundance (Hennes and Suttle, 1995). However, this method is also used to enumerate prokaryotes in the aquatic environment, and sometimes it is difficult to distinguish prokaryotes from viruses, namely in marine environment where prokaryotes are small and, consequently, pass through the 0.2 µm membranes used to concentrate prokaryotes, being concentrated on the 0.02 µm membranes used to concentrate viruses, as shown in tables 5 and 6.

The results of this study show that using epifluorescence microscopy some prokaryotes are counted as viruses, namely in the marine zone, overestimating viral density and underestimating prokaryotes density. In the estuarine system Ria de Aveiro 14 to 27% of the particles counted as viruses are prokaryotes. The FISH results using

probes for bacteria and archaea show that the majority of these prokaryotes counted as viruses are bacteria.

The common occurrence of lysogenic in Ria de Aveiro associated to the high numbers of lytic viruses and to the potential of these viruses to control bacterial density, activity and diversity, indicates that in Ria de Aveiro the virioplankton can have an important role in biogeochemical cycling of nutrients as well as in horizontal genetic exchange.

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